

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of

Koichi ISHIGURO, et al.

Application No. 08/56090

Filed: March 13, 1996

For: A METHOD FOR DIAGNOSING ALZHEIMER'S DISEASE



VERIFICATION OF TRANSLATION

Honorable Commissioner of Patents and Trademarks  
Washington, D.C. 20231

Sir:

Yoshiyuki KAWAGUCHI, residing at c/o SERA, TOYAMA, MATSUKURA & KAWAGUCHI, Yokoyama Building 6th Floor, 4-10, Higashi Nihonbashi 3-chome, Chuo-ku, Tokyo, 103 Japan, declares:

- (1) that I know well both Japanese and English languages;
- (2) that I translated the Priority Document (the basic Japanese Patent Application No. 8-56090 filed on March 13, 1996) from Japanese to English;
- (3) that the attached English translation is a true and correct translation of the priority document to the best of my knowledge and belief; and
- (4) that all statements made of his own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 USC 1001, and that such false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed at Tokyo, Japan, this 9th day of February, 2001.

Yoshiyuki KAWAGUCHI

[Document Name]        Application for patent  
[Number]                960171  
[Application filed]       March 13, 1996  
[To]                    Commissioner of Patent Office  
[International Class]     G01N 33/50  
[Title of the Invention]    A METHOD FOR DIAGNOSING  
ALZHEIMER'S DISEASE  
[Number of Claims]        6  
[Inventor]  
                         [Address]        C/O MITSUBISHI KASEI INSTITUTE OF  
LIFE SCIENCE, 11 Minamiooya, Machida-shi, Kanagawa  
                         [Name]            ISHIGURO Koichi  
[Inventor]  
                         [Address]        C/O MITSUBISHI KASEI INSTITUTE OF  
LIFE SCIENCE, 11 Minamiooya, Machida-shi, Kanagawa  
                         [Name]            SATO Kazuki  
[Inventor]  
                         [Address]        C/O MITSUBISHI KASEI INSTITUTE OF  
LIFE SCIENCE, 11 Minamiooya, Machida-shi, Kanagawa  
                         [Name]            PARK Jun-Mi  
[Inventor]  
                         [Address]        C/O MITSUBISHI KASEI INSTITUTE OF  
LIFE SCIENCE, 11 Minamiooya, Machida-shi, Kanagawa  
                         [Name]            UCHIDA Tsuneko  
[Inventor]  
                         [Address]        C/O MITSUBISHI KASEI INSTITUTE OF  
LIFE SCIENCE, 11 Minamiooya, Machida-shi, Kanagawa

[Name] IMAHORI Kazutomo  
[Applicant]  
[ID Number] 000005968  
[Name] MITSUBISHI CHEMICAL  
CORPORATION  
[Representative] MIURA Akira  
[Patent Attorney]  
[ID Number] 000005968  
[Patent Attorney]  
[Name] HASEGAWA Koji  
[Indication of Official Fees]  
[ledger number] 035035  
[amount paid] 21,000  
[List of attached documents]  
[document name] Specification 1  
[document name] Drawing 1  
[document name] Abstract 1  
[General power of attorney number] 9702254

[Document name]                      Specification

[Title of the invention] A METHOD FOR DIAGNOSING ALZHEIMER'S  
DISEASE

[Claims]

[Claim 1]    A method for diagnosing Alzheimer's disease by using an antibody obtained by using as an immunogen a partial peptide comprising a phosphorylated site of phosphorylated tau protein in a paired helical filament.

[Claim 2]    The method for diagnosing Alzheimer's disease according to claim 1, wherein the phosphorylated site of phosphorylated tau protein is one or more amino acid residues selected from serine at position 198, serine at position 199, serine at position 202, threonine at position 205, threonine at position 231, serine at position 235, serine at position 262, serine at position 396, threonine at position 403, serine at position 404, serine at position 409, serine at position 412, serine at position 413, and serine at position 422 of an amino acid sequence of SEQ ID NO: 1.

[Claim 3]    The method for diagnosing Alzheimer's disease according to claim 1, wherein the phosphorylated site of phosphorylated tau protein is one or more amino acid residues selected from serine at position 199, serine at position 202, threonine at position 205, threonine at position 231, serine at position 235, serine at position 262, serine at position 396, serine at position 404, serine at position 412, serine at position 413, and serine at position 422 of an amino acid sequence of SEQ ID NO: 1.

[Claim 4]    The method for diagnosing Alzheimer's disease

according to any one of claims 1 to 3, wherein the partial peptide comprising the phosphorylated site comprises an amino acid residue at the phosphorylated site and 3 - 5 amino acid residues at forward and backward sites of the phosphorylated site.

[Claim 5] The method for diagnosing Alzheimer's disease according to any one of claims 1 to 4, wherein the partial peptide comprising the phosphorylated site has an amino acid sequence of any one of SEQ ID NO: 2 to SEQ ID NO: 16.

[Claim 6] A reagent kit for diagnosing Alzheimer's disease by using an antibody obtained by using as an immunogen a partial peptide comprising a phosphorylated site of phosphorylated tau protein in a paired helical filament

[Detailed Description of the Invention]

[Technical Field of the Invention]

The present invention relates to a method for diagnosing Alzheimer's disease. More specifically, the present invention relates to a method for diagnosing Alzheimer's disease by using an antibody obtained by using as an immunogen a partial peptide comprising a phosphorylated site of phosphorylated tau protein in a paired helical filament.

[Prior Art and Problems to be Solved by the Invention]

Alzheimer's disease is progressive dementia occurring at the presenile stage (the age of 45 to 65). It causes morbid

changes such as degeneration of neurocytes and atrophia of cerebral cortex due to a decrease in the number of neurocytes. Pathologically, a number of senile plaques and neurofibrillary degeneration are observed in the brain. So-called senile dementia caused by spontaneous aging in the senium of the age of 65 or older is not substantially different from Alzheimer's disease from the pathological viewpoint and is regarded as Alzheimer 's senile dementia.

The number of patients suffering from Alzheimer 's disease increases as senile population grows. This disease has thus attracted social attention. There are various hypotheses about the causes of this disease. However, it has not been elucidated yet and it is desired to clarify soon.

The main component of senile plaques that is one of pathological changes caused by Alzheimer's disease is known to be amyloid  $\beta$  protein (Annu. Rev. Neurosci., 12, 463-490 (1989)). Neurofibrillary degeneration that is another pathological change shows accumulation of the paired helical filament in neurocytes and tau protein is identified as one of its constituents (J. Biochem. 99, 1807-1810 (1986); Proc. Natl. Acad. Sci. USA, 83, 4913-4917 (1986)).

Tau protein is composed of a group of analogous proteins that usually produce several bands at the molecular weight of 48 to 65 kD as a result of SDS-polyacrylamide gel electrophoresis and it is known to promote formation of microtubule. Tau protein incorporated in the PHF of the Alzheimer diseased brain was proved to be abnormally phosphorylated as compared with that in the normal brain using polyclonal antibody to PHF (anti-ptau;

J. Biochem., 99, 1807-1810) and monoclonal antibody to tau protein (tau-1 antibody; Proc. Natl. Acad. Sci. USA, 83, 4913-4917 (1986)). The phosphorylated site of phosphorylated tau protein incorporated in the PHF was also identified (Japanese Patent Application No.7-177241). Thus, functions of tau protein involved in Alzheimer's disease has being clarified.

However, it has not been known so far to diagnose Alzheimer's disease based on the phosphorylated site of phosphorylated tau protein in the PHF. Although methods for diagnosing Alzheimer's disease using various antibodies have been proposed, a clinically effective new diagnosing method is still desired.

[Means for Solving the Problems]

The present inventors paid attention to the phosphorylated site of phosphorylated tau protein in the PHF and found that antibodies obtained by using as an immunogen a partial peptide comprising a phosphorylated site is useful for detecting Alzheimer's disease, thereby completing the present invention.

The present invention provides a method for diagnosing Alzheimer's disease by using an antibody obtained by using as an immunogen a partial peptide comprising a phosphorylated site of phosphorylated tau protein in the paired helical filament.

Preferred embodiments of this invention provides:

the method as described above, wherein the phosphorylated site of phosphorylated tau protein is one or more amino acid residues selected from serine at position 198, serine at

position 199, serine at position 202, threonine at position 205, threonine at position 231, serine at position 235, serine at position 262, serine at position 396, threonine at position 403, serine at position 404, serine at position 409, serine at position 412, serine at position 413, and serine at position 422 of an amino acid sequence of SEQ ID NO: 1;

the method as described above, wherein the phosphorylated site of phosphorylated tau protein is one or more amino acid residues selected from serine at position 198, serine at position 199, serine at position 202, threonine at position 205, threonine at position 231, serine at position 235, serine at position 262, serine at position 396, serine at position 404, serine at position 409, serine at position 412, serine at position 413, and serine at position 422 of the amino acid sequence of SEQ ID NO: 1;

the method as described above, wherein the phosphorylated site of phosphorylated tau protein is one or more amino acid residues selected from serine at position 199, serine at position 202, threonine at position 205, threonine at position 231, serine at position 235, serine at position 262, serine at position 396, serine at position 404, serine at position 412, serine at position 413, and serine at position 422 of the amino acid sequence of SEQ ID NO: 1;

the method as described above, wherein the partial peptide comprising the phosphorylated site comprises an amino acid residue at the phosphorylated site and 3 - 5 amino acid residues at forward and backward sites of the phosphorylated site; and

the method as described above, wherein the partial peptide



comprising the phosphorylated site has an amino acid sequence of any one of SEQ ID NO: 2 to SEQ ID NO: 16.

Another embodiment of this invention provides a reagent kit for diagnosing Alzheimer's disease by using an antibody obtained by using as an immunogen a partial peptide comprising a phosphorylated site of phosphorylated tau protein in the paired helical filament.

[Embodiment of the Invention]

The present invention will be described in detail below.

In the present invention, an example of tau protein is that having a primary structure consisting of 352nd to 441st amino acid residues as described in Goedert et al., Neuron 3, 519-526 (1989). For example, when tau protein having the primary structure represented by the amino acid sequence shown in SEQ ID NO: 1 in the sequence listing, one or more amino acid residues to be phosphorylated are selected from serine at position 198, serine at position 199, serine at position 202, threonine at position 205, threonine at position 231, serine at position 235, serine at position 262, serine at position 396, threonine at position 403, serine at position 404, serine at position 409, serine at position 412, serine at position 413, and serine at position 422 of the same sequence (J. Biol. Chem., 270, 823-829 (1995) and Neurosci. Lett., 189, 167-170 (1995)).

It is preferable to use a partial peptide of tau protein comprising one or more phosphorylated amino acid residues selected from serine at position 199, serine at position 202,

threonine at position 205, threonine at position 231, serine at position 235, serine at position 262, serine at position 396, serine at position 404, serine at position 412, serine at position 413 and serine at position 422 of the above-described sequence.

A peptide comprising the amino acid residue(s) at the above-described phosphorylated site and 3 - 5 amino acid residues at forward and backward sites of the phosphorylated site(s) is preferably used as the partial peptide of tau protein used in the present invention, the peptide having the amino acid sequence described in any one of SEQ ID NO: 2 to 16 in the sequence listing is preferably used.

Among the above-described peptides, the partial peptides represented by SEQ ID NO: 3, 13, 15, and 16 are synthesized by the solid phase peptide synthesis using a phenyl group as a protective group for the phosphorylated site as described in Tetrahedron Lett., 32, 7083-7086 (1991).

The phosphorylated peptides represented by the other sequence than those represented by the above-described sequence identification number, which have an aromatic amino acid, a sulfur-containing amino acid, or a heterocyclic amino acid, are synthesized by the solid phase peptide synthesis using a cyclohexyl group as a protective group for the phosphorylated site as described in Peptide Chemistry 1993, 109-112 (1994) or by the solid phase peptide synthesis using a benzyl group as a protective group for the phosphorylated site as described in Chem. Lett., 1099-1112 (1994).

After the partial peptide synthesized as described above is bound to a carrier protein such as keyhole limpet hemocyanin, the immunization of an animal can be performed, and the antibody may be purified using a peptide column having a specific partial peptide immobilized to obtain an antibody specifically recognizing the above-described phosphorylated site. In the present invention, the thus-obtained antiserum can be used as it is without purification.

Alzheimer's disease can be diagnosed by immunologically reacting the antibody obtained by the above-described method with a sample derived from an individual suspected of Alzheimer's disease by per se known conventional methods, detecting an antigen-antibody reaction product, and examining reactivity between the sample and the antibody. The antibody obtained as described above can be used to prepare a reagent kit for detecting Alzheimer's disease by the above-described method comprising the immunoreaction and the detection step.

Alzheimer's disease can be detected using the antibody or the reagent kit of the present invention for example as follows.

A sample is first obtained from tissues, cerebrospinal fluid or blood, of an individual suspected of Alzheimer's disease and reacted with the antibody obtained as described above. As a tissue sample, cerebral cortex is used. When a tissue sample is subjected to the diagnosing method of the present invention, about 0.1 mg of the sample is required. When cerebrospinal fluid or blood is used as a sample, about 0.01 ml of the sample is required.

Once the above-described sample is obtained, the sample is

homogenized in physiological saline and centrifuged. The resulting supernatant is fractionated to remove contaminating immunoglobulin and examined for reactivity to the above-obtained antibody as an index.

The thus-obtained fraction is electrophoresed. The antibody as obtained above is added thereto to perform immunoblotting. In this occasion, the antibody can be detected by labeling it with the label used conventionally. Alternatively, the antibody may be detected by reacting it with a secondary antibody that is reactive with the antibody.

If a sample is examined for reactivity with the antibody and the reactivity increases compared with a control derived from an individual without Alzheimer's disease, the individual suspected of Alzheimer's disease is regarded to be a patient of Alzheimer's disease. If the reactivity decreases compared with a control from an individual with Alzheimer's disease, the individual suspected of Alzheimer's disease is regarded to be free from Alzheimer disease. In this way, Alzheimer's disease can be diagnosed by the present invention.

[Examples]

The present invention will be described below in more detail with reference to Examples, but is not construed to be limited thereto.

Production Example 1

Preparation of partial peptide having amino acid sequence

described in SEO ID NO: 3 in the sequence listing (This peptide and the antibody against this peptide are hereinafter sometimes referred to as "PS202" and "anti-PS202", respectively.)

A peptide shown by H-Lys-Ser-Ser-Pro-Gly-Ser( $\text{H}_2\text{PO}_3$ )-Pro-Gly-Thr-Pro-Gly-Ser-Arg- $\text{NH}_2$  was produced by the following method. Symbols hereinafter used respectively have the following meanings: MBHA resin: p-methylbenzhydrylamine resin; Boc: t-butyloxycarbonyl group; Bzl: benzyl group; Ph: phenyl group; Tos: p-toluenesulfonyl group; and Z(2-Cl): 2-chlorobenzyloxycarbonyl group.

(a) Production of H-Lys[Z(2-Cl)]-Ser(Bzl)-Ser(Bzl)-Pro-Gly-Ser[PO(OPh)<sub>2</sub>]-Pro-Gly-Thr(Bzl)-Pro-Gly-Ser(Bzl)-Arg(Tos)-MBHA resin

A 0.94 g of MBHA resin (amine content: 0.64 mmol/g resin) was set in the Bio Search 9500 Model Automatic Peptide Synthesizer. Boc-Arg(Tos)-OH, Boc-Ser(Bzl)-OH, Boc-Gly-OH, Boc-Pro-OH, Boc-Thr(Bzl)-OH, Boc-Gly-OH, Boc-Pro-OH, Boc-Ser[PO(OPh)<sub>2</sub>]-OH, Boc-Gly-OH, Boc-Pro-OH, Boc-Ser(Bzl)-OH, Boc-Ser(Bzl)-OH, and Boc-Lys[Z(2-Cl)]-OH were supplied thereto and were then joined in this order using diisopropylcarbodiimide as a condensing agent to obtain 2.38 g of the above-described side chain-protected peptide-MBHA resin.

(b) Treatment with hydrogen fluoride

A 1.34 g of the side chain-protected peptide-MBHA resin obtained in (a) was collected and set in a hydrogen fluoride reaction device manufactured by the Protein Research Recommending Association Peptide Research Laboratory. The resin was reacted with 13 ml of hydrogen fluoride in the presence of 1.5 ml anisole under ice-cooling for 1 hour. After the

completion of the reaction, hydrogen fluoride was distilled off under reduced pressure. The residue was washed with ethyl acetate and extracted with 150 ml of 2 M acetic acid to obtain 350 mg of a crude peptide having a protected phosphate group and shown by H-Lys-Ser-Ser-Pro-Gly-Ser[PO(OPh)<sub>2</sub>]-Pro-Gly-Thr-Pro-Gly-Ser-Arg-NH<sub>2</sub>.

This product was dissolved in 20 ml of 30% acetic acid and the resulting solution was applied to a Sephadex G-25 column (internal diameter: 5 cm and length: 109 cm). Elution was performed using the same solvent to collect the fraction containing the desired product. The thus-obtained fraction was then dissolved in a small amount of distilled water and purified by HPLC using a reverse phase column (internal diameter: 2 cm and length: 25 cm) packed with ODS (octadecylsilane)-bound silica. Elution was performed by a linear gradient of 5 to 65% acetonitrile in 0.1% trifluoroacetic acid. The yield of the purified product was 110 mg. The structure of this substance was confirmed by FAB mass spectrometry; measured  $[M + H]^+$ ; 1445, calculated (C<sub>61</sub>H<sub>92</sub>N<sub>18</sub>O<sub>21</sub>P<sub>1</sub> + H); 1445.

(c) Hydrogenolysis

90 mg of the phosphate group-protected peptide obtained in (b) and 80 mg of platinum oxide (catalyst) were mixed with 1 ml of acetic acid and the mixture was stirred at room temperature for 12 hours under hydrogen atmosphere of 5 to 6 pressure. After the catalyst was filtered off, the filtrate and washings were collected and lyophilized. The resulting product was purified by preparatory HPLC to obtain 55 mg of a final product, phosphorylated peptide shown by H-Lys-Ser-Ser-Pro-Gly-Ser[H<sub>2</sub>PO<sub>3</sub>]-

Pro-Gly-Thr-Pro-Gly-Ser-Arg-NH<sub>2</sub>. The structure of this substance was confirmed by FAB mass spectrometry; measured [M + H]<sup>+</sup>; 1294, calculated (C<sub>49</sub>H<sub>85</sub>N<sub>18</sub>O<sub>21</sub>P<sub>1</sub> + H); 1294.

#### Production Examples 2 to 4

Partial peptides having amino acid sequences described in SEQ ID NO: 13, NO: 15, and NO: 16 in the sequence listing were obtained in the same manner as described in Production Example 1 (These peptides are hereinafter sometimes referred to as "PS413", "PS412", and "PS412, 413" and the antibodies to these peptides are referred to as "anti-PS413", "anti-PS412", and "anti-PS412, 413", respectively.)

#### Production Example 5

Preparation of partial peptide having amino acid sequence described in SEQ ID NO: 2 in the sequence listing (This peptide and an antibody to this peptide are hereinafter sometimes referred to as "PS199" and "anti-PS199", respectively.)

A peptide shown by H-Lys-Ser-Gly-Tyr-Ser-Ser(H<sub>2</sub>PO<sub>3</sub>)-Pro-Gly-Ser-Pro-Gly-Thr-NH<sub>2</sub> was produced by the following method. Symbols used hereinafter respectively have the following meanings: MBHA resin: p-methylbenzhydrylamine resin; Boc: t-butyloxycarbonyl group; Bzl: benzyl group; cHex: cyclohexane group; Z(2-Br): 2-bromobenzyloxycarbonyl group; and Z(2-Cl): 2-chlorobenzyloxycarbonyl group.

(a) Production of H-Lys[Z(2-Cl)]-Ser(Bzl)-Gly-Tyr[Z(2-Br)]-Ser(Bzl)-Ser[PO(OcHex)<sub>2</sub>]-Pro-Gly-Ser(BzL)-Pro-Gly-Thr(Bzl)-MBHA resin

A 131 mg of MBHA resin (amine content: 0.76 mmol/g resin)

was set in the Bio Search 9500 Model Automatic Peptide Synthesizer. Boc-Thr(Bzl)-OH, Boc-Gly-OH, Boc-Pro-OH, Boc-Ser(Bzl)-OH, Boc-Gly-OH, Boc-Pro-OH, Boc-Ser[PO(OcHex)<sub>2</sub>]-OH, Boc-Ser(Bzl)-OH, Tyr[Z(2-Br)]-OH, Boc-Gly-OH, Boc-Ser(Bzl)-OH, and Boc-Lys[Z(2-Cl)]-OH were supplied thereto and were then joined in this order using diisopropylcarbodiimide as a condensing agent to obtain 307 mg of the above-described side chain-protected peptide-MBHA resin.

(b) Treatment with trifluoromethanesulfonic acid

A 150 mg of the side chain-protected peptide-MBHA resin obtained in (a) was collected. 10 ml of trifluoroacetic acid containing 1 M methanesulfonic acid and thioanisole, and 0.05 ml of m-cresol were added thereto and the resulting mixture was allowed to react for 4 hours under ice-cooling. After the completion of the reaction, 200 ml of ice-cooled diethyl ether was added thereto to precipitate a peptide. The whole content was collected by filtration with a glass filter and washed with cold diethyl ether. The solution was extracted with 200 ml of 2 M acetic acid to obtain 53 mg of a crude peptide shown by H-Lys-Ser-Gly-Tyr-Ser-Ser[H<sub>2</sub>PO<sub>3</sub>]-Pro-Gly-Ser-Pro-Gly-Thr-NH<sub>2</sub>.

(c) Purification of the peptide

This product was dissolved in 6 ml of distilled water and purified by HPLC using a reverse phase column (internal diameter: 2 cm and length: 25 cm) packed with ODS (octadecylsilane)-bound silica. Elution was performed by a linear gradient of 5 to 35% acetonitrile in 0.1% trifluoroacetic acid. The yield of the purified product was 29 mg. The structure of this substance was confirmed by FAB mass



spectrometry; measured  $[M + H]^+$ ; 1204, calculated  $(C_{47}H_{75}N_{14}O_{21}P_1 + H)$ ; 1204.

Production Example 6

Preparation of partial peptide having amino acid sequence described in SEQ ID NO: 6 in the sequence listing (This peptide and an antibody to this peptide are hereinafter sometimes referred to as "PT231" and "anti-PT231", respectively.)

A peptide shown by H-Cys-Val-Ala-Val-Val-Arg-Thr( $H_2PO_3$ )-Pro-Pro-Lys-Ser-Pro-Ser-Ser-OH was produced by the following method. Symbols used hereinafter respectively have the following meanings: Bzl resin: benzyl alcohol resin; Boc: t-butyloxycarbonyl group; Bzl: benzyl group; MBzl: 4-methoxybenzyl group; Mts: methylenesulfonyl group; cHex: cyclohexyl group; and Z(2-Cl): 2-chlorobenzoyloxycarbonyl group.

(a) Production of H-Cys(MBzl)-Val-Ala-Val-Val-Arg(Mts)-Thr[PO(OcHex)<sub>2</sub>]-Pro-Pro-Lys[Z(2-Cl)]-Ser(Bzl)-Pro-Ser(Bzl)-Ser(Bzl)-Bzl resin

71 mg of Boc-Ser(Bzl)-Bzl resin (amine content: 0.70 mmol/g resin) was set in the Bio Search 9500 Model Automatic Peptide Synthesizer. Boc-Ser(Bzl)-OH, Boc-Pro-OH, Boc-Ser(Bzl)-OH, Boc-Lys[Z(2-Cl)]-OH, Boc-Pro-OH, Boc-Pro-OH, Boc-Thr[PO(OcHex)<sub>2</sub>]-OH, Boc-Arg(Mts)-OH, Boc-Val-OH, Boc-Val-OH, Boc-Ala-OH, Boc-Val-OH, and Boc-Cys(MBzl)-OH were supplied thereto and were then joined in this order using diisopropylcarbodiimide as a condensing agent to obtain 62 mg of the above-described side chain-protected peptide-Bzl resin.

(b) Treatment with trifluoromethanesulfonic acid

To 62 mg of the side chain-protected peptide-Bzl resin obtained in (a) were added 0.9 ml of trifluoromethanesulfonic acid, 1.2 ml of thioanisole, 6.6 ml of trifluoroacetic acid, 0.9 ml of m-cresol, and 0.4 ml of ethanedithiol. The mixture was allowed to react for 5 minutes under ice-cooling and then at room temperature for 3 hours. After the completion of the reaction, 200 ml of ice-cooled diethyl ether was added thereto to precipitate a peptide. The whole content was collected by filtration with a glass filter and washed with cold diethyl ether. The solution was extracted with 170 ml of 2 M acetic acid to obtain 21 mg of a crude peptide shown by H-Cys-Val-Ala-Val-Val-Arg-Thr( $\text{H}_2\text{PO}_3$ )-Pro-Pro-Lys-Ser-Pro-Ser-Ser-OH.

(c) Purification of the peptide

This product was dissolved in 10 ml of 30% acetic acid and applied to a Sephadex G-25 column (internal diameter: 5 cm and length: 107 cm). Elution was performed using the same solvent to collect the fractions containing the desired product. The yield of this purified product was 12 mg.

The thus-obtained product was dissolved in 5 ml of 30% acetic acid and purified by HPLC using a reverse phase column (internal diameter: 2 cm and length: 25 cm) packed with ODS (octadecylsilane)-bound silica. Elution was performed with 13% acetonitrile in 0.1% trifluoroacetic acid. The yield of the purified product was 7 mg. The structure of this substance was confirmed by FAB mass spectrometry; measured  $[M + H]^+$ ; 1509, calculated ( $\text{C}_{61}\text{H}_{107}\text{N}_{18}\text{O}_{22}\text{P}_1\text{S}_1 + \text{H}$ ): 1508.

Production Example 7

Preparation of partial peptide having amino acid sequence described in SEQ ID NO: 11 in the sequence listing (This peptide and an antibody to this peptide are hereinafter sometimes referred to as "PS396" and "anti-PS396", respectively.)

A peptide shown by H-Cys-Glu-Ile-Val-Tyr-Lys-Ser( $H_2PO_3$ )-Pro-Val-Val-Ser-Gly-NH<sub>2</sub> was produced by the following method. Symbols used hereinafter respectively have the following meanings: MBHA resin: p-methylbenzhydrylamine resin; Boc: t-butyloxycarbonyl group; Bzl: benzyl group; MBzl, 4-methoxybenzyl group; cHex: cyclohexyl group; Z(2-Br): 2-bromobenzyloxycarbonyl group; and Z(2-Cl): 2-chlorobenzyloxycarbonyl group.

(a) Production of H-Cys(MBzl)-Glu(OBzl)-Ile-Val-Tyr[Z(2-Br)]-Lys[Z(2-Cl)]-Ser[PO(OcHex)<sub>2</sub>]-Pro-Val-Val-Ser(Bzl)-Gly-MBHA resin

A 131 mg of MBHA resin (amine content: 0.76 mmol/g resin) was set in the Bio Search 9500 Model Automatic Peptide Synthesizer. Boc-Gly-OH, Boc-Ser(Bzl)-OH, Boc-Val-OH, Boc-Val-OH, Boc-Pro-OH, Boc-Ser[PO(OcHex)<sub>2</sub>]-OH, Boc-Lys[Z(2-Cl)]-OH, Tyr[Z(2-Br)]-OH, Boc-Val-OH, Boc-Ile-OH, Boc-Glu(OBzl)-OH, and Boc-Cys(MBzl)-OH were supplied thereto and were then joined in this order using diisopropylcarbodiimide as a condensing agent to obtain 376 mg of the above-described side chain-protected peptide-MBHA resin.

(b) Treatment with trifluoromethanesulfonic acid

To 188 mg of the side chain-protected peptide-MBHA resin obtained in (a) were added 10 ml of trifluoroacetic acid containing 1 M methanesulfonic acid and thioanisole, and 0.05 ml of m-cresol. The mixture was allowed to react for 4 hours under ice-cooling. After the completion of the reaction, 200 ml of

ice-cooled diethyl ether was added thereto to precipitate a peptide. The whole content was collected by filtration with a glass filter and washed with cold diethyl ether, and extracted with 200 ml of 2 M acetic acid to obtain 87 mg of a crude peptide shown by H-Cys-Glu-Ile-Val-Tyr-Lys-Ser(H<sub>2</sub>PO<sub>3</sub>)-Pro-Val-Val-Ser-Gly-NH<sub>2</sub>.

(c) Purification of the peptide

This product was dissolved in 9 ml of 30% acetic acid and purified by HPLC using a reverse phase column (internal diameter: 2 cm and length: 25 cm) packed with ODS (octadecylsilane)-bound silica. Elution was performed with 16% acetonitrile in 0.1% trifluoroacetic acid. The yield of the purified product was 45 mg. The structure of this substance was confirmed by FAB mass spectrometry; measured  $[M + H]^+$ ; 1360, calculated (C<sub>57</sub>H<sub>95</sub>N<sub>14</sub>O<sub>20</sub>P<sub>1</sub>S<sub>1</sub> + H); 1360.

Production Examples 8 to 10

Partial peptides having amino acid sequence described in SEQ ID NO: 4, NO: 12, and NO: 14 in the sequence listing were obtained in the same manner as in Production Examples 5, 6, and 7 (These peptides are hereinafter referred to as "PT205", "PS404", and "PS422", respectively, and antibodies to these peptides are referred to as "anti-PT205", "anti-PS404", and "anti-PS422", respectively.)

Production Example 11

Preparation of partial peptide having amino acid sequence described in SEQ ID NO: 7 in the sequence listing (This peptide

and an antibody to this peptide are hereinafter sometimes referred to as "PS235" and "anti-PS235", respectively.)

A peptide shown by H-Cys-Arg-Thr-Pro-Pro-Lys-Ser( $\text{H}_2\text{PO}_3$ )-Pro-Ser-Ser-Ala-Lys-OH was produced by the following method. Symbols used hereinafter respectively have the following meanings: Alko resin: p-alkoxybenzyl alcohol resin; Boc: t-butyloxycarbonyl group; tBu: t-butyl group; Bzl: benzyl group; Fmoc: 9-fluorenylmethoxycarbonyl group; Trt: trityl group; Pmc: pentamethylchroman-6-sulfonyl group.

(a) Production of H-Cys(Trt)-Arg(Pmc)-Thr(tBu)-Pro-Pro-Lys(Boc)-Ser[PO(OH)(OBzl)]-Pro-Ser(tBu)-Ser(tBu)-Ala-Lys(Boc)-Alko resin

A 385 mg of Fmoc-Lys(Boc)-Alko resin (amino acid content: 0.65 mmol/g resin) was set in the Applied Bio Systems 431 Model Automatic Peptide Synthesizer. Fmoc-Ala-OH, Fmoc-Ser(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Pro-OH, Fmoc-Ser[PO(OH)(OBzl)]-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Pro-OH, Fmoc-Thr(tBu)-OH, and Fmoc-Arg(Pmc)-OH were supplied thereto and were then joined in this order using HBTU [2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] as a condensing agent to obtain 716 mg of a side chain-protected peptide-Alko resin intermediate. Fmoc-Cys(Trt)-OH was condensed with 358 mg of this intermediate to obtain 395 mg of the above-described side chain-protected peptide-Alko resin.

(b) Treatment with trifluoroacetic acid

To 196 mg of the side chain-protected peptide-Alko resin obtained in (a) was added a mixture containing 8.25 ml of trifluoroacetic acid, 0.5 ml of purified water, 0.5 ml of thioanisole, 0.75 ml of phenol, and 0.25 ml of ethanedithiol.

The resulting mixture was allowed to react for 1.5 hour at room temperature. After the completion of the reaction, 200 ml of ice-cooled diethyl ether was added thereto to precipitate a peptide. The whole content was collected by filtration with a glass filter, washed with cold diethyl ether, and extracted with 80 ml of 2 M acetic acid to obtain 82 mg of a crude peptide shown by H-Cys-Arg-Thr-Pro-Pro-Lys-Ser(H<sub>2</sub>PO<sub>3</sub>)-Pro-Ser-Ser-Ala-Lys-OH.

(c) Purification of the peptide

This product was dissolved in 7 ml of 0.1% trifluoroacetic acid and purified by HPLC using a reverse phase column (internal diameter: 2 cm and length: 25 cm) packed with ODS (octadecylsilane)-bound silica. Elution was performed with 7% acetonitrile in 0.1% trifluoroacetic acid. The yield of the purified product was 62 mg. The structure of this substance was confirmed by FAB mass spectrometry; measured  $[M + H]^+$ ; 1339, calculated (C<sub>52</sub>H<sub>92</sub>N<sub>17</sub>O<sub>20</sub>P<sub>1</sub>S<sub>1</sub> + H); 1339.

Production Examples 12 to 15

Partial peptides having amino acid sequence described in SEQ ID NO: 5, NO: 8, NO: 9, and NO: 10 in the sequence listing were obtained in the same manner as in Production Example 11 (These peptides are hereinafter sometimes referred to as "PS199,202", "ratPS235", "PT231,PS235", and "PS262", respectively, and antibodies to these peptides are referred to as "anti-PS199,202", "anti-ratPS235", "anti-PT231,PS235", and "anti-PS262", respectively.)

Production Example 16

Preparation of partial peptide having amino acid sequence described in SEQ ID NO: 17 in the sequence listing (This peptide and an antibody to this peptide are hereinafter sometimes referred to as "Tau-C" and "anti-Tau-C", respectively.)

A peptide shown by H-Ser-Pro-Gln-Leu-Ala-Thr-Leu-Ala-Asp-Glu-Val-Ser-Ala-Ser-Leu-Ala-Lys-OH was produced by the following method. Symbols used hereinafter respectively have the following meanings: Alko resin: p-alkoxybenzyl alcohol resin; Boc: t-butyloxycarbonyl group; tBu: t-butyl group; Bzl: benzyl group; Fmoc: 9-fluorenylmethoxycarbonyl group; and Trt: trityl group.

(a) Production of H-Ser(tBu)-Pro-Gln(Trt)-Leu-Ala-Thr(tBu)-Leu-Ala-Asp(OtBu)-Glu(OtBu)-Val-Ser(tBu)-Ala-Ser-Leu-Ala-Lys(Boc)-Alko resin

A 284 mg of Alko resin (amine content: 0.88 mmol/g resin) was set in the ABI A431 Model Automatic Peptide Synthesizer. Fmoc-Lys(Boc)-OH was bound to the resin using dimethylaminopyridine and diisopropylcarbodiimide as condensing agents. Then, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Ser(tBu)-OH, Fmoc-Ala-OH, Fmoc-Ser(tBu)-OH, Fmoc-Val-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Thr(tBu)-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Gln(Trt)-OH, Fmoc-Pro-OH, and Fmoc-Ser(tBu)-OH were supplied thereto and were then joined in this order using HBTU [2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] as a condensing agent to obtain 905 mg of the above-described side chain-protected peptide-Alko resin.

(b) Treatment with trifluoroacetic acid

To 543 mg of the side chain-protected peptide-Alko resin obtained in (a) were added 9.5 ml of trifluoroacetic acid, 0.25 ml of ethanedithiol, and 0.5 ml of distilled water. The mixture was allowed to react for 5 minutes under ice-cooling and then at room temperature for 1.5 hour. After the completion of the reaction, 200 ml of ice-cooled diethyl ether was added thereto to precipitate a peptide. The whole content was collected by filtration with a glass filter, washed with cold diethyl ether, and extracted with 100 ml of 2 M acetic acid to obtain 250 mg of a crude peptide shown by H-Ser-Pro-Gln-Leu-Ala-Thr-Leu-Ala-Asp-Glu-Val-Ser-Ala-Ser-Leu-Ala-Lys-OH.

(c) Purification of the peptide

This product was dissolved in 20 ml of 30% acetic acid and applied to a Sephadex G-25 column (internal diameter: 5 cm and length: 107 cm). Elution was performed using the same solvent to collect the fractions containing the desired product. The yield of this purified product was 122 mg. The structure of this substance was confirmed by FAB mass spectrometry; measured  $[M + H]^+$ ; 1702, calculated  $(C_{73}H_{125}N_{19}O_{27}S_2 + H)$ ; 1701.

Production Example 17

Preparation of partial peptide having amino acid sequence described in SEQ ID NO: 18 in the sequence listing (This peptide and an antibody to this peptide are hereinafter sometimes referred to as "Tau-N" and "anti-Tau-N", respectively.)

A peptide shown by H-Ala-Glu-Pro-Arg-Gln-Glu-Glu-Phe-Glu-Val-Met-Glu-Cys-NH<sub>2</sub> was produced by the following method.



Symbols used hereinafter respectively have the following meanings: Fmoc-NH-SAL resin: 4-(2',4'-dimethoxyphenyl-Fmoc-aminoethyl)phenoxy resin; Boc: t-butyloxy-carbonyl group; tBu: t-butyl group; Bzl: benzyl group; Fmoc: 9-fluorenylmethoxycarbonyl group; Trt: trityl group; and Pmc: pentamethylchroman-6-sulfonyl group.

(a) Production of H-Ala-Glu(OtBu)-Pro-Arg(Pmc)-Gln(Trt)-Glu(OtBu)-Phe-Glu(OtBu)-Val-Met-Glu(OtBu)-Cys(Trt)-NH-SAL resin

A 532 mg of Fmoc-NH-SAL resin (amine content: 0.47 mmol/g resin) was set in the ABI A431 Model Automatic Peptide Synthesizer. Fmoc-Cys(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Met-OH, Fmoc-Val-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Phe-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Pro-OH, Fmoc-Glu(OtBu)-OH, and Fmoc-Ala-OH were supplied thereto and were then joined in this order using HBTU [2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] as a condensing agent to obtain 1122 mg of the above-described side chain-protected peptide-NH-SAL resin.

(b) Treatment with trifluoroacetic acid

To 673 mg of the side chain-protected peptide-NH-SAL resin obtained in (a) were added 0.75 ml of phenol, 0.5 ml of thioanisole, 8.25 ml of trifluoroacetic acid, 0.25 ml of ethanedithiol, and 0.5 ml of distilled water. The mixture was allowed to react for 5 minutes under ice-cooling and then at room temperature for 1.5 hour. After the completion of the reaction, 200 ml of ice-cooled diethyl ether was added thereto to precipitate a peptide. The whole content was collected by filtration with a glass filter, washed with cold diethyl ether,

and extracted with 50 ml of 2 M acetic acid and 250 ml of distilled water to obtain 182 mg of a crude peptide shown by H-Ala-Glu-Pro-Arg-Gln-Glu-Glu-Phe-Glu-Val-Met-Glu-Cys-NH<sub>2</sub>.

(c) Purification of the peptide

This product was dissolved in 20 ml of 30% acetic acid and applied to a Sephadex G-25 column (internal diameter: 5 cm and length: 107 cm). Elution was performed using the same solvent to collect the fractions containing the desired product. The yield of this purified product was 136 mg.

This product was dissolved in 20 ml of 20% acetonitrile and purified by HPLC using a reverse phase column (internal diameter: 2 cm and length: 25 cm) packed with ODS (octadecylsilane)-bound silica. Elution was performed with 22% acetonitrile in 0.1% trifluoroacetic acid. The yield of the purified product was 96 mg. The structure of this substance was confirmed by FAB mass spectrometry; measured  $[M + H]^+$ ; 1467, calculated (C<sub>61</sub>H<sub>95</sub>N<sub>17</sub>O<sub>21</sub>S<sub>2</sub> + H); 1467.

Production Example 18

Preparation of antibody

The partial peptides obtained in Production Examples 1 to 17 were each bound to an equivalent weight of keyhole limpet hemocyanin to serve as an immunogen, rabbits were immunized. The thus-obtained antisera was purified by applying onto a Affigel 15 column (Bio-Rad) to which the respective antigenic peptide was bound and eluted using Immunopure gentle Ag/Ab buffer system (Pierce). Thus, the above-described antibodies that specifically recognize the respective phosphorylated sites

were obtained.

Specificity of the antibodies was confirmed by dot blotting.

More specifically, immunobilon P-membrane (Millipore) was dotted with 18 pmol of each peptide dissolved in a 70% DMSO solution in line with each other, allowed to adsorb them, and dried. This membrane was immersed in TBS (20 mM Tris-HCl (pH 7.5), 150 mM NaCl) containing 5% skim milk for 1 hour to prevent non-specifically binding of the antibodies to be added in the following step to the membrane. The membrane was then washed with TBS for 5 minutes three times, immersed in TBS containing the desired antibody (first antibody), placed between parafilm, and allowed to react at 4°C for 14 hours in a humidified box to bind the first antibodies to the respective antigen protein on the membrane. The membrane was then washed with TBS containing 0.05% Tween 20 (TBST) for 5 minutes three times. The following procedure until color development was performed using ProtoBlot Western Blot AP System (Promega).

Anti-rabbit IgG antibody to which alkaline phosphatase was covalently bound (second antibody) was diluted 5000-fold with TBST. The membrane was immersed in this diluted solution at 4°C for 2 hours to bind alkaline phosphatase to the antigen-first antibody binding product on the membrane through the second antibody. The membrane was washed with TBST for 5 minutes three times, then with TBS for 5 minutes twice. The membrane was immersed in a reaction mixture (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM MgCl<sub>2</sub>) supplemented with 0.165 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 0.33 mg/ml of nitro blue

tetrazolium (NBT) and the existence of alkaline phosphatase on the membrane was detected by development of purple color. The reaction was terminated by immersing the membrane in water.

The first antibodies used in this Example were all rabbit antisera as they were. The same results were obtained using IgG obtained by purifying the antisera by affinity chromatography using a peptide column. The dilution of each antiserum was 1000-fold for anti-PS199, 250-fold for anti-PS202, 500-fold for anti-PT205, 250-fold for anti-PT231, 250-fold for anti-PS235, 25-fold for anti-rat PS235 (anti-rPS235), 500-fold for anti-PS262, 1000-fold for anti-PS396, 500-fold for anti-PS404, 500-fold for anti-PS413, and 500-fold for anti-PS422. The control peptides used were unphosphorylated peptides including the amino acid sequence shown by SEQ ID NO: 19 (a peptide shown by K1, the 226th to the 240th amino acid residues, in SEQ ID NO: 1 in Fig. 1), the amino acid sequence shown by SEQ ID NO: 20 (a peptide shown by K2, the 191st to the 224th amino acid residues, in SEQ ID NO: 1 in Fig. 1), the amino acid sequence shown by SEQ ID NO: 21 (a peptide shown by AK-K3, the 384th to the 438th amino acid residues, in SEQ ID NO: 1 in Fig. 1), and the amino acid sequence shown by SEQ ID NO: 22 (a peptide shown by S262, the 257th to the 267th amino acid residues having a cysteine residue at the N-terminus, in SEQ ID NO: 1 in Fig. 1). These peptides were produced in the same manner in (a) and (b) in Production Example 1.

The results of dot blotting are shown in Fig. 1. The abscissa shows the peptide adsorbed by dotting it on the membrane and the ordinate shows the antibody. It shows that the

respective antibodies obtained as described above are specifically bound to the corresponding phosphorylated sites.

### Example 1

#### Diagnosis of Alzheimer's disease by using antibody

##### (1) Preparation of human brain extract

Human brain extracts were prepared from 8 cases of normal human brains and 19 cases of human brains of patients with Alzheimer's disease. The following procedure was all performed at 4°C.

1 g was sampled from chilled specimen of postmortem human cerebral cortex, cut into pieces with a razor in 3 ml of a TSinh solution [50 mM Tris-HCl (pH 7.6), 0.15 M NaCl, 0.5 mM DIFP (diisopropylfluorophosphate), 1 µg/ml antipain, 0.5 mM PMSF (phenylmethanesulfonyl fluoride), 1 mg/ml of TLCK (tricyl-lysine-chloromethyl ketone), 1 µg/ml of leupeptin, 0.1 g/ml of pepstatin], sonicated, and made into a suspension with a homogenizer. The resulting suspension was centrifuged at 80,000 rpm for 15 minutes to obtain a supernatant. Human IgG in this supernatant was removed with Protein G-Sepharose 4 Fast Flow (Pharmacia) and the resulting fraction was designated as TS fraction. The precipitate was sonicated in 2 ml of the above-described TSinh solution and made into a suspension with a homogenizer. The resulting suspension was washed by centrifugation at 80,000 rpm for 15 minutes and the thus-obtained precipitate was sonicated in 2 ml of TSinh containing

1% Triton X-100 and made into a suspension with a homogenizer. The homogenized product was centrifuged at 80,000 rpm for 15 minutes to obtain a supernatant (TX fraction). The precipitate was sonicated in 2 ml of TSinh containing 1% Triton X-100, made into a suspension with a homogenizer and washed by centrifugation at 80,000 rpm for 15 minutes. The resulting precipitate was sonicated in 2 ml of TSinh containing 2% SDS and homogenized to obtain a suspension. The suspension was centrifuged at 80,000 rpm for 15 minutes to obtain a supernatant (SDS supernatant fraction). The resulting precipitate was sonicated in 2 ml of TSinh containing 2% SDS, made into a suspension with a homogenizer, and washed by centrifugation at 80,000 for 15 minutes. The thus-obtained precipitate was sonicated in 2 ml of TSinh containing 2% SDS and made into a suspension (SDS precipitate fraction) with a homogenizer.

(2) Immunoblotting using phosphorylated site-specific antibodies

Laemmli's sample treatment solution (Nature 227, 680-685 (1970)) was added to each fraction obtained above. The mixture was heated at 95°C for 5 minutes and subjected to SDS polyacrylamide gel electrophoresis. The resulting electrophoretic patterns were transferred to Immunobilon P-membrane (Millipore). After blocking with TBS containing 5% skim milk for 2 hours, the membrane was reacted with the phosphorylated site-specific antibody obtained in Example 1 as a first antibody for 14 hours. The reaction mixture was washed with Tween 20 and TBS and treated with ProtoBlot AP (Promega) as a second antibody to develop color of the antibody-antigen reaction product on the membrane.

The first antibody was all rabbit IgG and dilution of the first antibody was shown by the following numerals after "x" under the names of the respective antibodies in Figs. 2 to 4 with respect to Examples. Almost all of the first antibodies were previously purified by affinity chromatography using an antigen peptide column. However, P262 and PS422 were used in the form of antisera and distinguished from others by indicating "S" under the name of antibodies in these figures.

Tau-N and Tau-C are peptides obtained in Production Examples 16 and 17 and correspond to the 2nd to the 12th and the 422nd to the 438th amino acid residues, respectively, of human tau protein represented by SEQ ID NO: 1 in the sequence listing, and used as controls showing the existence of tau protein.

A positive control used was whole brain extract of 8-day-old juvenile rat. Juvenile tau protein is known to be highly phosphorylated tau protein in the PHF (J. Biol. Chem. 268, 25712-25717 (1993)). Specifically, 0.75 g of 8-day-old juvenile rat brain was homogenized in 1.5 ml of medium containing 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 50 mM  $\beta$ -glycerophosphoric acid,  $10^{-4}$  M  $\text{Na}_3\text{VO}_4$ , 1 mM PMSF, 1  $\mu\text{g}/\text{ml}$  antipain, 1  $\mu\text{g}/\text{ml}$  leupeptin, 0.1  $\mu\text{g}/\text{ml}$  pepstatin, and 3 mM benzamidine. The homogenized product was centrifuged at 25,000 rpm for 20 minutes and a supernatant was recovered to serve as a positive control.

The results are shown in Figs. 2, 3, and 4. Figure 2 shows the result of immunoblotting of the TS fraction; Figures 3 and 4 show the result of immunoblotting of the SDS precipitate fraction. In these figures, "A" in lane AD/N stands for the

result of brain extract from an Alzheimer's disease patient; " N ", for the result of normal brain extract; " M ", for molecular weight markers; the next numerals for the molecular weight of the markers in terms of kD. Rat P8 lane stands for the immunoblotting result of the positive control and the band indicated by an arrow is phosphorylated tau protein band.

Any antibody did not react with the normal brain extract, but did react with the brain extract from an Alzheimer's disease patient. This indicates that the method of the present invention enables diagnosing Alzheimer's disease. The TS fraction contains easily soluble tau protein, whereas the SDS precipitation fraction contains less soluble tau protein. The method of the present invention enables detecting not only phosphorylated tau protein in tissues but also that in cerebrospinal fluid or blood thought to contain easily soluble tau protein. In other words, cerebrospinal fluid and blood can also be used as a sample as well as tissues.

#### [Effect of the Invention]

The present invention provides diagnosing Alzheimer's disease by using an antibody that specifically recognizes phosphorylated tau protein using as an immunogen a partial peptide containing a phosphorylated site of phosphorylated tau protein in the PHF.



**SEQUENCE :**

Met	Ala	Glu	Pro	Arg	Gln	Glu	Phe	Glu	Val	Met	Glu	Asp	His	Ala	Gly
1				5					10					15	
Gln	Asp	Thr	Tyr	Gly	Leu	Gly	Asp	Arg	Lys	Asp	Gln	Gly	Gly	Tyr	Thr
			20					25					30		
Met	His	Gln	Glu	Gly	Asp	Thr	Asp	Ala	Gly	Leu	Lys	Glu	Ser	Pro	Leu
		35					40					45			
Gln	Thr	Pro	Thr	Glu	Asp	Gly	Ser	Glu	Glu	Pro	Gly	Ser	Glu	Thr	Ser
	50					55				60					
Asp	Ala	Lys	Ser	Thr	Pro	Thr	Ala	Glu	Asp	Val	Thr	Ala	Pro	Leu	Val
65					70					75					80
Asp	Glu	Gly	Ala	Pro	Gly	Lys	Gln	Ala	Ala	Ala	Gln	Pro	His	Thr	Glu
				85					90					95	
Ile	Pro	Glu	Gly	Thr	Thr	Ala	Glu	Glu	Ala	Gly	Ile	Gly	Asp	Thr	Pro
		100						105				110			
Ser	Leu	Glu	Asp	Glu	Ala	Ala	Gly	His	Val	Thr	Gln	Ala	Arg	Met	Val
		115					120					125			
Ser	Lys	Ser	Lys	Asp	Gly	Thr	Gly	Ser	Asp	Asp	Lys	Lys	Ala	Lys	Gly
	130					135					140				
Ala	Asp	Gly	Lys	Thr	Lys	Ile	Ala	Thr	Pro	Arg	Gly	Ala	Ala	Pro	Pro
145					150					155					160
Gly	Gln	Lys	Gly	Gln	Ala	Asn	Ala	Thr	Arg	Ile	Pro	Ala	Lys	Thr	Pro
				165					170					175	
Pro	Ala	Pro	Lys	Thr	Pro	Pro	Ser	Ser	Gly	Glu	Pro	Pro	Lys	Ser	Gly
			180					185					190		
Asp	Arg	Ser	Gly	Tyr	Ser	Ser	Pro	Gly	Ser	Pro	Gly	Thr	Pro	Gly	Ser
		195					200					205			
Arg	Ser	Arg	Thr	Pro	Ser	Leu	Pro	Thr	Pro	Pro	Thr	Arg	Glu	Pro	Lys
	210					215					220				
Lys	Val	Ala	Val	Val	Arg	Thr	Pro	Pro	Lys	Ser	Pro	Ser	Ser	Ala	Lys
225					230					235					240
Ser	Arg	Leu	Gln	Thr	Ala	Pro	Val	Pro	Met	Pro	Asp	Leu	Lys	Asn	Val
				245					250					255	
Lys	Ser	Lys	Ile	Gly	Ser	Thr	Glu	Asn	Leu	Lys	His	Gln	Pro	Gly	Gly
			260					265					270		

Gly	Lys	Val	Gln	Ile	Ile	Asn	Lys	Lys	Leu	Asp	Leu	Ser	Asn	Val	Gln
		275					280					285			
Ser	Lys	Cys	Gly	Ser	Lys	Asp	Asn	Ile	Lys	His	Val	Pro	Gly	Gly	Gly
		290				295					300				
Ser	Val	Gln	Ile	Val	Tyr	Lys	Pro	Val	Asp	Leu	Ser	Lys	Val	Thr	Ser
305					310					315					320
Lys	Cys	Gly	Ser	Leu	Gly	Asn	Ile	His	His	Lys	Pro	Gly	Gly	Gly	Gln
				325					330					335	
Val	Glu	Val	Lys	Ser	Glu	Lys	Leu	Asp	Phe	Lys	Asp	Arg	Val	Gln	Ser
			340					345					350		
Lys	Ile	Gly	Ser	Leu	Asp	Asn	Ile	Thr	His	Val	Pro	Gly	Gly	Gly	Asn
		355				360						365			
Lys	Lys	Ile	Glu	Thr	His	Lys	Leu	Thr	Phe	Arg	Glu	Asn	Ala	Lys	Ala
		370				375					380				
Lys	Thr	Asp	His	Gly	Ala	Glu	Ile	Val	Tyr	Lys	Ser	Pro	Val	Val	Ser
385					390					395					400
Gly	Asp	Thr	Ser	Pro	Arg	His	Leu	Ser	Asn	Val	Ser	Ser	Thr	Gly	Ser
				405					410					415	
Ile	Asp	Met	Val	Asp	Ser	Pro	Gln	Leu	Ala	Thr	Leu	Ala	Asp	Glu	Val
			420					425					430		
Ser	Ala	Ser	Leu	Ala	Lys	Gln	Gly	Leu							
		435					440								

SEQ ID NO:2:

SEQUENCE LENGTH: 12

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

FEATURE:

OTHER INFORMATION : Xaa=phosphoserine

SEQUENCE:

Lys	Ser	Gly	Tyr	Ser	Xaa	Pro	Gly	Ser	Pro	Gly	Thr
1				5					10		

SEQ ID NO:3:

SEQUENCE LENGTH: 13

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

FEATURE:

OTHER INFORMATION : Xaa=phosphoserine

SEQUENCE:

Lys Ser Ser Pro Gly Xaa Pro Gly Thr Pro Gly Ser Arg  
1 5 10

SEQ ID NO:4:

SEQUENCE LENGTH: 12

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

FEATURE:

OTHER INFORMATION : Xaa=phosphothreonine

SEQUENCE:

Cys Pro Gly Ser Pro Gly Xaa Pro Gly Ser Arg Ser  
1 5 10

SEQ ID NO:5:

SEQUENCE LENGTH: 13

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

FEATURE:

OTHER INFORMATION : Xaa=phosphoserine

SEQUENCE:

Lys Ser Xaa Pro Gly Xaa Pro Gly Thr Pro Gly Ser Arg  
1 5 10

SEQ ID NO:6:

SEQUENCE LENGTH: 14

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

FEATURE:

OTHER INFORMATION : Xaa=phosphothreonine

SEQUENCE:

Cys Val Ala Val Val Arg Xaa Pro Pro Lys Ser Pro Ser Ser  
1 5 10

SEQ ID NO:7:

SEQUENCE LENGTH: 12

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

FEATURE:

OTHER INFORMATION : Xaa=phosphoserine

SEQUENCE:

Cys Arg Thr Pro Pro Lys Xaa Pro Ser Ser Ala Lys  
1 5 10

SEQ ID NO:8:

SEQUENCE LENGTH: 12

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

FEATURE:

OTHER INFORMATION : Xaa=phosphoserine

SEQUENCE:

Cys Arg Thr Pro Pro Lys Xaa Pro Ser Ala Ser Lys  
1 5 10

SEQ ID NO:9:

SEQUENCE LENGTH: 12

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

FEATURE:

OTHER INFORMATION : Xaa=phosphothreonine

SEQUENCE:

Cys Arg Xaa Pro Pro Lys Xaa Pro Ser Ser Ala Lys  
1 5 10

SEQ ID NO:10:

SEQUENCE LENGTH: 12

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

FEATURE:

OTHER INFORMATION : Xaa=phosphoserine

SEQUENCE:

Cys Lys Ser Lys Ile Gly Xaa Thr Glu Asn Leu Lys  
1 5 10

SEQ ID NO:11:

SEQUENCE LENGTH: 12

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

FEATURE:

OTHER INFORMATION : Xaa=phosphoserine

SEQUENCE:

Cys Glu Ile Val Tyr Lys Xaa Pro Val Val Ser Gly  
1 5 10

SEQ ID NO:12:

SEQUENCE LENGTH: 12

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

FEATURE:

OTHER INFORMATION : Xaa=phosphoserine

SEQUENCE:

Cys Val Ser Gly Asp Thr Xaa Pro Arg His Leu Ser  
1 5 10

SEQ ID NO:13:

SEQUENCE LENGTH: 12

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

FEATURE:

OTHER INFORMATION : Xaa=phosphoserine

SEQUENCE:

Lys Leu Ser Asn Val Ser Xaa Thr Gly Ser Ile Asp  
1 5 10

SEQ ID NO:14:

SEQUENCE LENGTH: 12

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

FEATURE:

OTHER INFORMATION : Xaa=phosphoserine

SEQUENCE:

Cys Ile Asp Met Val Asp Xaa Pro Gln Leu Ala Thr  
1 5 10

SEQ ID NO:15:

SEQUENCE LENGTH: 12

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

FEATURE:

OTHER INFORMATION : Xaa=phosphoserine

SEQUENCE:

Lys Leu Ser Asn Val Xaa Ser Thr Gly Ser Ile Asp  
1 5 10

SEQ ID NO:16:

SEQUENCE LENGTH: 12

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

FEATURE:

OTHER INFORMATION : Xaa=phosphoserine

SEQUENCE:

Lys Leu Ser Asn Val Xaa Xaa Thr Gly Ser Ile Asp  
1 5 10

SEQ ID NO:17:

SEQUENCE LENGTH: 17

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

SEQUENCE:

Ser Pro Gln Leu Ala Thr Leu Ala Asp Glu Val Ser Ala Ser Leu Ala Lys  
1 5 10 15

SEQ ID NO:18:

SEQUENCE LENGTH: 12

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

SEQUENCE:

Ala Glu Pro Arg Gln Glu Phe Glu Val Met Glu Cys  
1 5 10

SEQ ID NO:19:

SEQUENCE LENGTH: 15

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

SEQUENCE: SEQ ID NO:19:

Val Ala Val Val Arg Thr Pro Pro Lys Ser Pro Ser Ser Ala Lys  
1 5 10 15

SEQ ID NO:20:

SEQUENCE LENGTH: 34

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

SEQUENCE:

Ser Gly Asp Arg Ser Gly Tyr Ser Ser Pro Gly Ser Pro Gly Thr Pro  
1 5 10 15  
Gly Ser Arg Ser Arg Thr Pro Ser Leu Pro Thr Pro Pro Thr Arg Glu  
20 25 30  
Pro Lys

SEQ ID NO:21:

SEQUENCE LENGTH: 55

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

SEQUENCE:

Ala Lys Thr Asp His Gly Ala Glu Ile Val Tyr Lys Ser Pro Val Val  
1 5 10 15  
Ser Gly Asp Thr Ser Pro Arg His Leu Ser Asn Val Ser Ser Thr Gly  
20 25 30  
Ser Ile Asp Met Val Asp Ser Pro Gln Leu Ala Thr Leu Ala Asp Glu  
35 40 45  
Val Ser Ala Ser Leu Ala Lys  
50 55

SEQ ID NO:22:

SEQUENCE LENGTH: 12

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: peptide

SEQUENCE: SEQ ID NO:22:

Cys Lys Ser Lys Ile Gly Ser Thr Glu Asn Leu Lys  
1 5 10

Brief Description of the Drawings

Figure 1 is the dot blot showing specificity of the antibodies obtained by immunization with a partial peptide containing a phosphorylated site of phosphorylated tau protein.

5

Figure 2 is photographs of electrophoresis (immunoblotting) showing reactivity of the TS fraction obtained in Example with the antibodies used in the present invention.

10

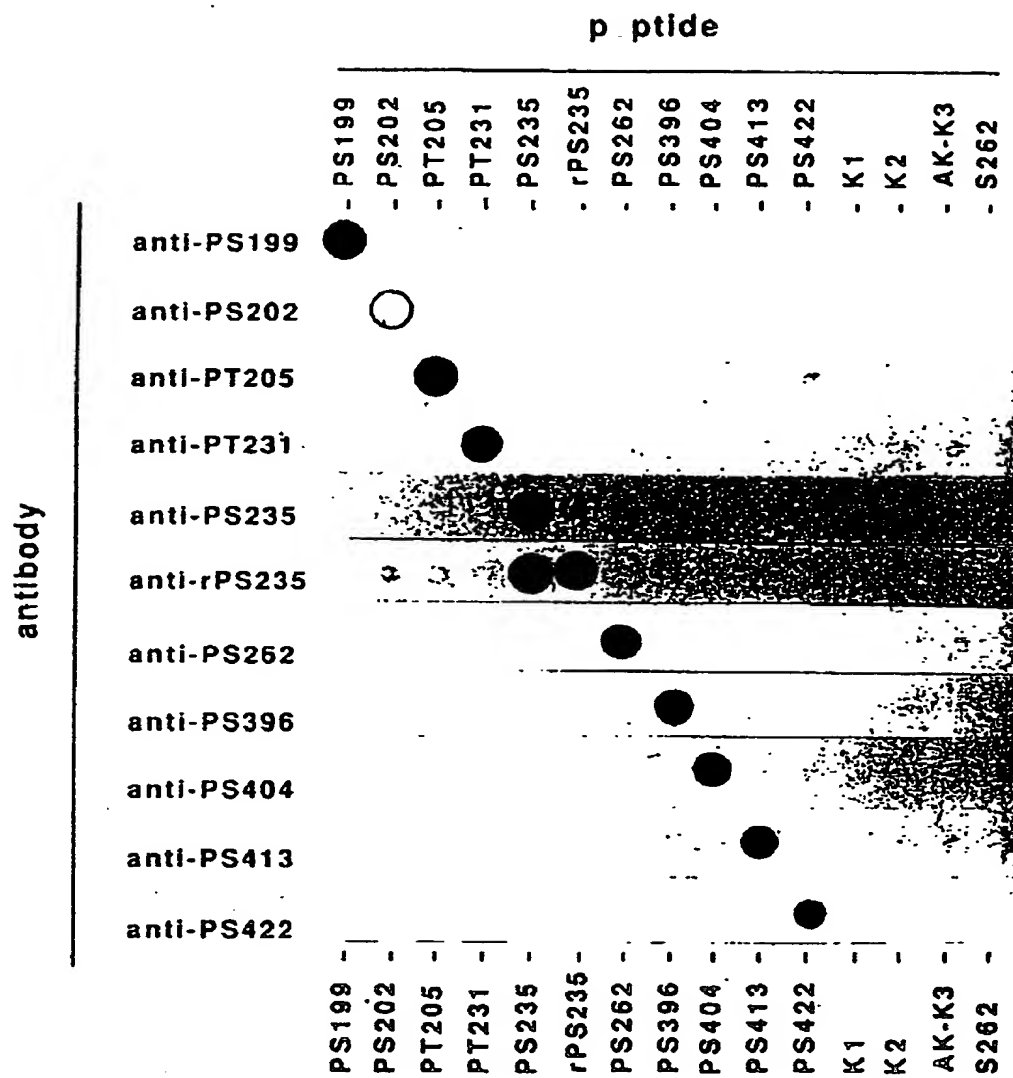
Figure 3 is photographs of electrophoresis (immunoblotting) showing reactivity of the SDS precipitation fraction obtained in Example with the antibodies used in the present invention.

15

Figure 4 is photographs of electrophoresis (immunoblotting) showing reactivity of the SDS precipitation fraction obtained in Example with the antibodies used in the present invention.

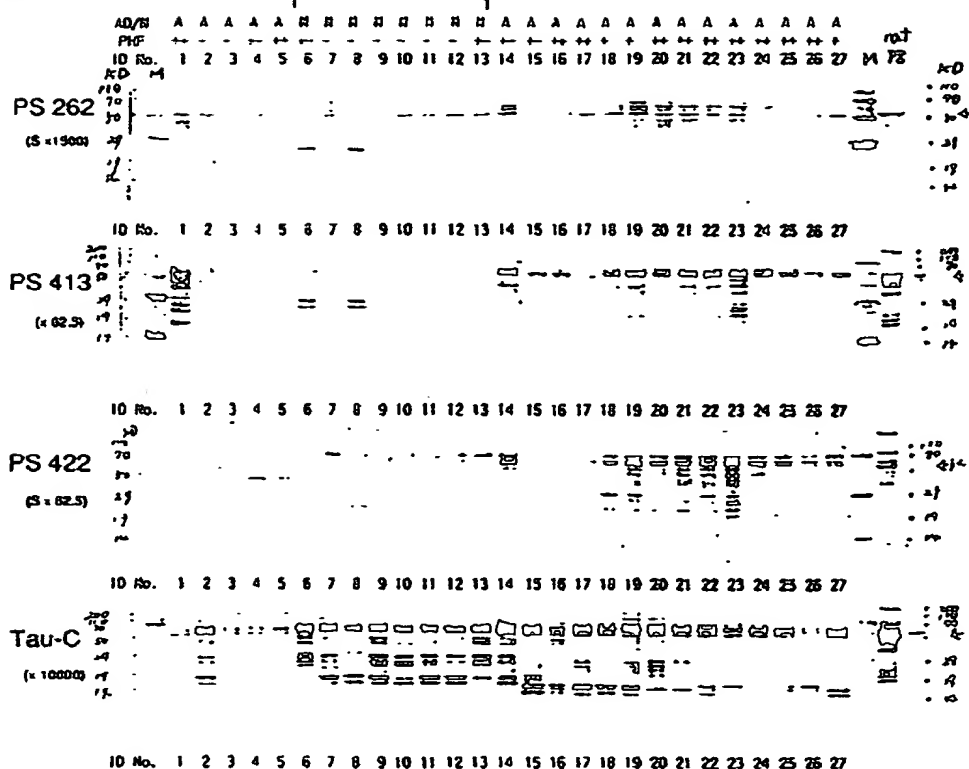


[Document Name] Drawing  
 [Figure 1]

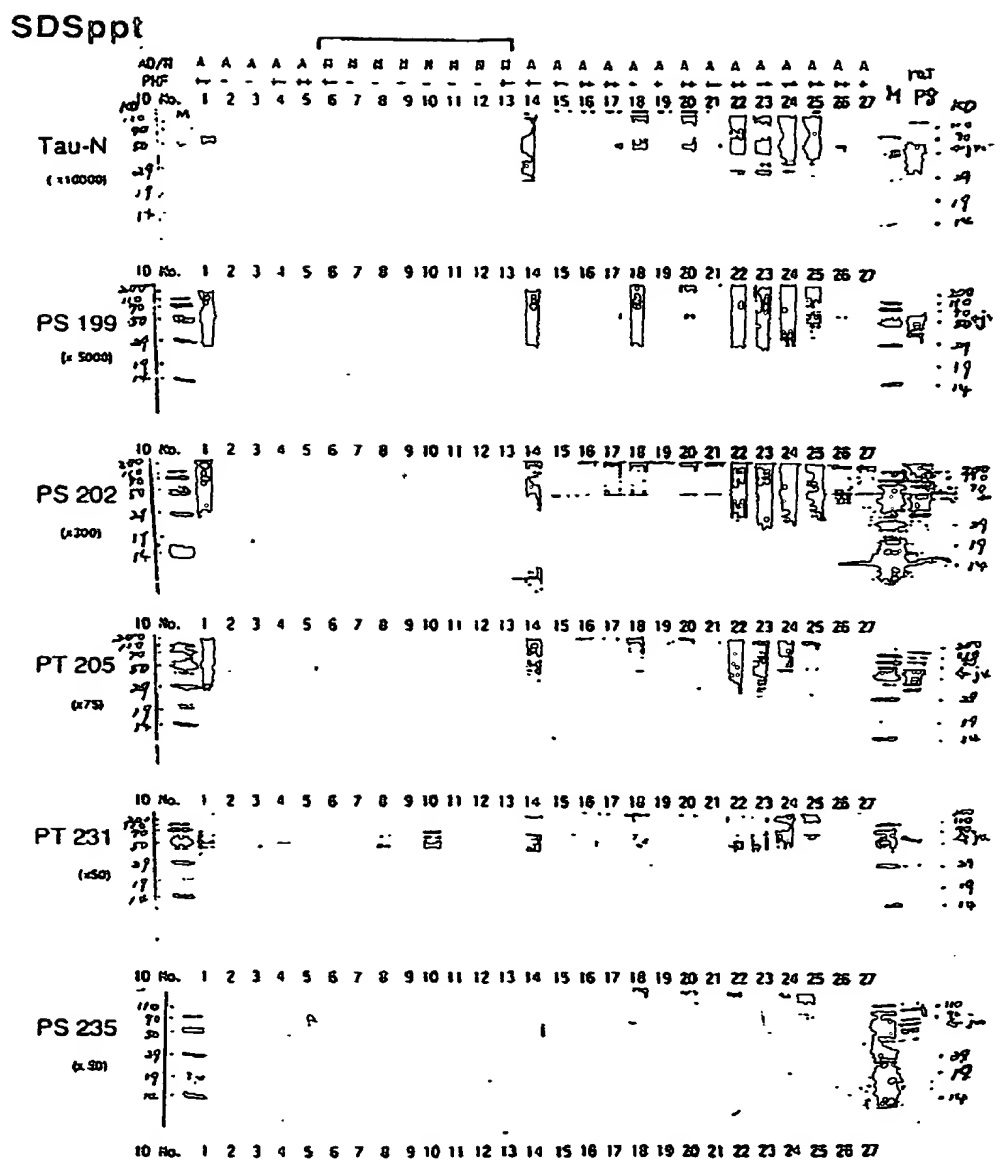


[Figure 2]

TS

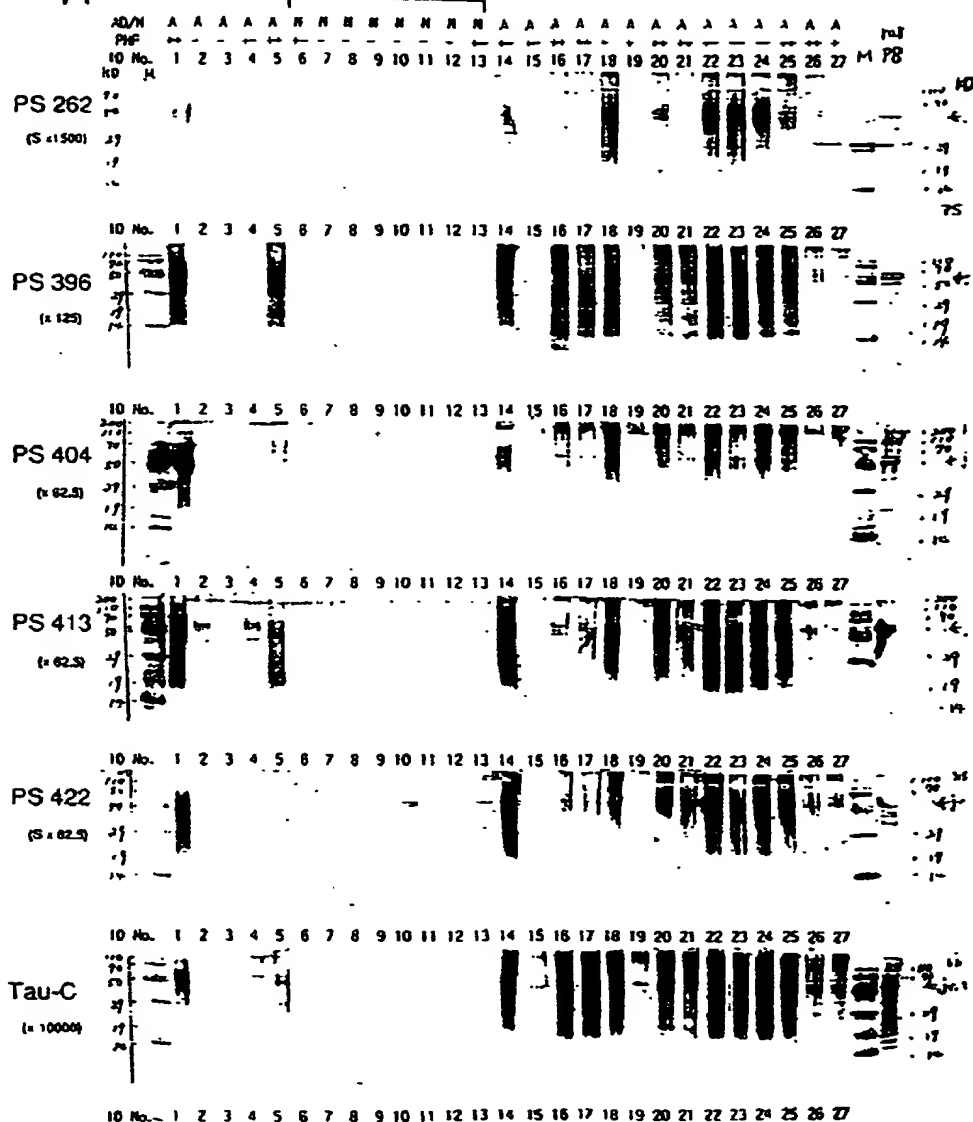


A black and white micrograph showing a single cell. The cell has a large, dark, oval-shaped nucleus in the center, surrounded by a lighter cytoplasm. The cell membrane is visible as a thin, irregular border.



[Figure 4]

# SDSppt



[Document Name]            Abstract

[Abstract]

[Object]     To provide a clinically effective  
method for diagnosing Alzheimer's disease

[construction]     A method for diagnosing Alzheimer's  
disease by using an antibody obtained by using as an  
immunogen a partial peptide comprising a  
phosphorylated site of phosphorylated tau protein in  
a paired helical filament.

[Selected Figure]            None